as a model protein; one is wild type (WT HO) and the other is a mutant (KDMY HO) which involves replacements of four residues (K16N, D27A, M31K, Y112F). Their X-ray structures indicated that the 4-vinyl of WT HO seems to have the same environments as that of 2-vinyl of KDMY in crystal. The NMR spectra confirmed the 90° rotation of the heme in solution, and their catalytic activities demonstrated the corresponding differences in the α and δ cleavages of the heme. The resonance Raman spectra of WT HO and KDMY HO are alike regarding major heme skeletal modes, whereas some modes involving heme side chains vary in frequencies and behaviors. To characterize such bands, we reconstituted the two proteins with vinyl- or propionate-deuterated hemes and compared their resonance Raman spectra in their oxidized, reduced, and CO-bound forms. The vinyl associated modes exhibited frequency shifts between the two proteins.

3I0936 ヘムオキシゲナーゼにおける CO 光解離後の構造変化および CO の放出経路

Protein motions and CO migration following CO photolysis in heme oxygenase

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Heme oxygenase (HO) catalyzes the physiological degradation of heme using dioxygen and reducing equivalents to produce biliverdin, ferrous ion, and CO. The HO reaction proceeds without severe product inhibition by CO generated in the second step of the HO reaction, even though CO is known to be a potent inhibitor of HO and other heme proteins. Therefore HO must have an efficient mechanism for discriminating between dioxygen and CO.

To explore protein motions and CO migration in HO following CO photolysis which mimics CO generation during the HO reaction, we collected X-ray diffraction data on the CO-heme-HO complex before and after continuous illumination by CW laser at temperatures below 160 K. The weighted difference Fourier map shows that CO was partially photolyzed from the heme iron, and photodissociated CO had migrated to a hydrophobic cavity, as observed in previous results at 35 K. In contrast to previous results, motions of the distal helix, water, and heme were observed which are the reverse of motions observed in CO binding. Notably, similar motions were absent in dioxygen binding and photolysis. These results imply that the CO-bound form is severely constrained by steric hindrance between the distal helix and CO bound to the heme iron, and that steric hindrance contributes to discrimination between binding of CO and dioxygen. Further, another putative CO trapping site, more polar than previous sites, was found. Combining these studies with previous results, we will discuss CO migration pathways in HO.

3I0948 多様な生物種によるヘムオキシゲナーゼ反応の微調整戦略：オキシヘムの安定化

Strategy of a variety of organisms for the fine-tuning of heme oxygenase reactions: Stabilization of the oxyheme intermediate

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Heme oxygenase (HO) catabolizes regioselective heme-ring opening to produce biliverdin, free iron, and carbon monoxide. In a variety of biological species, the HO reactions participate in recycling of iron, signaling, supplying resources of photoreceptive-molecule production, or generating anti-oxidants. To attain their own requirements for HO, each organism seems to take unique fine-tuning strategy. The HO reaction proceeds successively by consuming oxygen molecules and electrons, so that their constant supply is essential. In addition, formation of the first reactive species, hydroperoxy hemin, requires proton as well as electron transfer to the oxyheme. In different species, HO functions in different milieu, thereby regulated finely and differently by respective active sites. Our HO study is focused on such fine-tuning modes taken in different HOs from different species. In this study, stability of oxyheme, the first converted form of hemin in HO, was compared among the five HO complexes: rat HO1, puffer-fish HO1, cyanobacterial HO1, soybean HO1, and pathogenic-bacterial HO. The auto-oxidation rates of the oxyheme complexes showed unexpectedly big difference, which correlate with pKa of the heme-bound water as well as with g-agisotropy of the hydroxide-coordinated heme. Based on the crystal structures of three heme-HO complexes out of the five, very-well conserved Gly residue in the distal helix, closest to the heme iron, is proposed as the HB donor to stabilize the oxyheme in the heme pockets.

3I1010 Ultraviolet Resonance Raman Study on Indoleamine 2, 3-Dioxygenase

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Indoleamine 2, 3-Dioxygenase (IDO) catalyzes incorporation reaction of two oxygen atoms into L-tryptophan (Trp), generating N-formylkynurenine, which is in the major catabolic pathway of Trp in mammals. Visible resonance Raman (RR) studies identified a key intermediate of ferryl-oxo (Fe=O) species. This suggested that two oxygen atoms were transferred to substrate one by one. The Fe=O species seemed to lie in the compound II oxidation level and a question arose whether such an intermediate could perform oxo-transfer reaction, since no example had been reported. Such a reaction might be possible if the substrate intermediate is unusually reactive. In the present study, we have adopted ultraviolet (UV) RR spectroscopy to characterize the substrate bound to IDO, since the technique selectively enhances vibrational spectra of aromatic molecules. UVR scattering was excited at 229 nm for the ferrous state of IDO. UVR spectrum of substrate-free IDO was primarily contributed by Trp and Tyr residues. In the spectrum of the substrate-bound IDO, intensity of the W3 band of Trp was increased. This was ascribed either to bound-Trp or Trp residue in IDO. In the latter case, binding of Trp induced structural change of intrinsic Trp in IDO. In order to distinguish these possibilities, we utilized indole-2-¹³C- and indole-D₅-labeled Trp. The results showed that bound-Trp and not the intrinsic Trp exhibited the intensity change. The present study provides a new method to study the structure of the bound-substrate to IDO.

3I1022 An Intermediate Conformation of Cytochrome Oxidase in the Ligand-Free State Identified by Time-Resolved Resonance Raman Spectroscopy

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Cytochrome *c* oxidase (CcO) reduces dioxygen to water by heme *a*₃ and drives proton pump by heme *a*. In the present study, we applied green-pump and violet-probe time-resolved resonance Raman (TRRR) spectroscopy to monitor the dynamics of hemes after CO-photolysis with a time resolution of 10 ns. The green and violet light were used for CO-photolysis and resonance Raman excitation, respectively. TRRR spectra after CO-photolysis were measured at delay time = 10 ns - 5 ms. The Fe-His stretching mode was located at 221 wavenumbers at delay time between 10 ns and 100 ns, and then decreased exponentially with time with a time constant of 600 ns and at 5 ms reached 215 wavenumbers, which was the same value for equilibrium ligand-free form. This behavior was similar to that reported earlier. In this study, we found that intensity of the same mode decreased to two thirds at 100 ns relative to that at 10 ns and remained constant up to 5 ms, but was significantly smaller than the intensity for equilibrium ligand-free form. Results indicate that an intermediate conformation exists after ligand dissociation at delay time = 0.002 to 5 ms. This conformation might have a significantly smaller affinity to dioxygen compared with the conformation seen at 10 - 100 ns as judged from the Fe-His stretching frequency. The affinity control should be important to adjust the timing of dioxygen binding appropriately relative to the timing of proton loading for an efficient proton pumping.

3I1034 ポンプ・プローブレーザーと同期したパルスフローシステムの開発とそれを用いたタンパク質の時間分解赤外分光解析

Time-Resolved IR Analyses of Proteins Using a Novel Pulse Flow System Synchronized with Pump/Probe Lasers

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IR spectroscopy is a method to analyze structures and reactivities of functional groups, and powerful to reveal protein reaction mechanisms. This spectroscopy, however, has the crucial shortcoming that the application to aqueous solution is practically impossible in most cases because of strong IR absorption of water, which has prevented time-resolved (TR) IR measurements using a flow cell. Recently, we developed a new pump-probe TRIR spectrometer based on the femtosecond IR technology and using a spinning cell. Our spectrometer can detect a 30 μ OD protein signal even in the presence of $\sim$ 2 OD water background in 50 sec of data accumulation, and is promising for flow-flash TRIR measurements. Here, we developed a novel flow system that can be coupled

with the pump-probe TRIR spectrometer. A flow cell was composed of two CaF₂ windows and a 50-μm-thick Teflon spacer that has a 500-μm-wide observation channel. A syringe pump delivered protein solution to the cell by triggering with a pulse generator at 1 kHz (or less). The pulse generator was also used to trigger the pump/probe lasers at 1 kHz with an appropriate delay. The repetition rate of pump illumination can be prescaled with an optical chopper when needed to see slow protein dynamics. The system was tested on CO-bound cytochrome *c* oxidase (0.5 mM) at a flow rate of 1 ml/min. High performance of our flow system was shown through the observation of nanosecond-TR Fe_{a3}-CO and Cu_B-CO signals upon CO photolysis. Details will be discussed at the meeting.

3I1046 チトクロム *c* 酸化酵素のヘム *a* 側鎖に由来する共鳴ラマン線の帰属
The assignment of the resonance Raman bands of heme *a* peripheral substituents in cytochrome *c* oxidase

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Proton-pumping process coupled to the O₂ reduction reaction in cytochrome *c* oxidase (CcO) generates the electrochemical potential of proton across the membrane. The putative proton-pumping pathway in bovine heart CcO has H-bond with heme *a* substituents, namely, the formyl, the 7-propionate and the hydroxyfarnesylethyl (HFE) groups, and the proton-pumping mechanism driven by heme *a* is proposed. The functions of heme peripheries are remained to be elucidated and the structural changes of them are needed to be observed. Resonance Raman (RR) spectroscopy has a potential to do it and assignments of the vibrational modes are necessary.

RR band of the formyl group has already been assigned and those of the 7-propionate and the HFE groups are investigated in this study using bacterial mutants. Although another proton-pumping pathway is proposed in bacterial CcO, the structure of the redox centers and the RR spectra are almost identical to the bovine heart CcO. Y406 and T50 have hydrogen bonds with the 7-propionate and the HFE groups, and Y406F and T50A mutants show frequency shifts of the RR bands at 364 and 1251 cm⁻¹, respectively. These mutants have normal absorption spectra and cytochrome *c* oxidation activities, suggesting minimal effects of the mutations. The former band is assigned to the bending mode of the 7-propionate group and we propose an assignment of the vibrational mode of the HFE group to the 1251 cm⁻¹ band. The present study would enable us to monitor the heme peripheral groups during proton-pumping process.

3I1058 シアン結合型構造から推察される一酸化窒素還元酵素の反応機構
Functional Implications from Structural Characterization of CN-bound Nitric Oxide Reductase

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Denitrification, conversion of nitrate to N₂, is an example of anaerobic respiration. Nitric oxide reductase (NOR) catalyzes reduction of NO to N₂O; 2NO + 2H⁺ + 2e⁻ => N₂O + H₂O, a key step for denitrification. NOR is supposed to share the same progenitor with aerobic cytochrome *c* oxidase (COX) catalyzing four electron reduction of O₂, based on the structural similarity. While we know the structures of NOR and COX, structural factors determining their catalytic properties are still unknown. Here, we characterized the detailed structure of NOR, and compared the results with that of COX, to elucidate the structural elements controlling the function of NOR. The X-ray structure of oxidized NOR indicated that the active site structures of NOR and COX are similarly composed of heme iron and non-heme metal, while the non-heme metal is different between NOR and COX; Fe in NOR is replaced with Cu in COX. To get insights into the structural property of NOR, we utilized CN-bound form as a model for the substrate, NO, bound form. Spectroscopic data showed that CN-bound to heme did not interact with non-heme iron in oxidized NOR, contrasted with CN bridging heme and Cu in COX. Since binding of two NO to the active site is required for the NOR catalysis, contrary to one O₂ binding in COX, the CN ligand for heme in NOR avoid to interact with non-heme Fe to keep second substrate binding site. We will present the X-ray structure of CN-bound NOR and further discuss the determinants for the functional difference between NOR and COX.